Diapause in Tarnished Plant Bug (Heteroptera: Miridae) Reared in Dynamic Photoperiod Environmental Cabinets[1]

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ABSTRACT Diapause in tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois) (Heteroptera: Miridae), increased with decreasing photoperiods simulated in environmental cabinets to match those at Starkville, Mississippi (latitude 33.45 N). Hypertrophied fat body in males was strongly associated with hypotrophied accessory glands, and these two characteristics were used to classify male diapause. Although the fatty tissue surrounding the testes differentiated diapausing and reproductive males, almost all males had well-developed testes and large quantities of sperm in their seminal vesicles at the time of dissection; thus testis size was not used for diapause classification. Hypertrophied fat body and lack of mature eggs at time of dissection were used to classify female diapause. Simulated decreasing photoperiods beginning on 4 September, 17 September, and 1 October (Julian dates 247, 260, and 274, respectively) resulted in 15.4%, 57.0%, and 86.0% diapause, respectively. Males and females exhibited similar percentages of diapause. When only egg-producing females were considered, fewer eggs were present at time of dissection within females with the largest fat bodies than within females with smaller fat bodies. Diapause morphology was rated numerically and an SAS program was developed to classify diapausing and reproductive insects. Extrapolation from current data indicated that 50% of nymphs eclosing from eggs oviposited near Starkville, Mississippi, about 14 September can be expected to enter diapause.

KEY WORDS Overwinter, diapause, cotton insect, Miridae, Heteroptera

Tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois), and the western species, *Lygus hesperus* Knight, were the second most damaging pests of cotton in the United States, infesting 55% of cotton acreage and combining to reduce yields by 0.98% (≈300,000 bales; Williams 2002). In the midsouth region of the USA, tarnished plant bugs overwintering on preferred host plants near cotton fields tend to enter those fields as suitability of the preferred hosts diminishes

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(Snodgrass et al. 1984). The probability of yield losses increases as the tarnished plant bug population increases.

Despite the importance of this pest, little research has been done on its diapause and the condition of tarnished plant bugs in diapause. Greater knowledge of its diapause physiology may allow the attack of this phase of in the life cycle as a means of controlling this pest before it enters cotton fields and without the risk of inducing insecticide resistance. For example, one research project involves timing the release of sterile tarnished plant bugs to coincide with diapause termination in bugs overwintering on alternate hosts (Villavaso et al. 2002). Tarnished plant bug females in diapause are unmated and could be expected to mate upon terminating diapause. Two-hundred such females captured on goldenrod and held individually on green beans laid a total of 640 eggs, none of which hatched (G.L.S., unpublished).

Photoperiod plays a dominant role in diapause induction. The critical photoperiod is that which elicits diapause in 50% of a population (Tauber et al. 1986). Diapause in the heteropterans *Orius insidiosus* (Say) (Heteroptera: Anthocoridae) (Ruberson et al. 2000), *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae) (Hodek 1968), and *Graphosoma lineatum* (L.) (Heteroptera: Pentatomidae) (Nakamura et al. 1996) is regulated by photoperiod with diapausing *P. apterus* females having undeveloped ovaries (Hodek 1968). Prediapause oviposition has been rarely observed in *P. apterus* females (Hodek 1971), but not observed in two pentatomids, *Aelia acuminata* (L.) (Hodek 1974) and *G. lineatum* (Nakamura et al. 1996). An early laboratory study of tarnished plant bugs indicated that fixed photoperiods of 12.5 h of light or less per day induced nearly 100% diapause, and 14 h of light per day prevented diapause (Bariola 1969), but critical photoperiod was not mentioned. More recently, nymphs were collected from wild host plants between August and November near Stoneville, Mississippi (latitude 33.43 N) and reared to adulthood at a constant temperature (25 ± 4°C) near a window that provided natural light (Snodgrass 2003). Fifty percent of adults reared from the nymphs collected during the second week of September exhibited diapause characteristics. The date on which the critical photoperiod occurred was estimated as 12 September. The 12.5 h sunrise-to-sunset photoperiod on that date was reported to be the critical photoperiod (Snodgrass 2003); however, that photoperiod did not take into account the additional 50 min of light between civil dawn and sunrise and between sunset and civil dusk. Those periods of low light intensity are known to affect diapause induction (Chapman 1969, Tauber et al. 1986). Additionally, the study used bugs of unknown age and, because only natural photoperiods were available, the same cohort of bugs could be tested for only one dynamic photoperiod.

Fixed photoperiods do not occur in nature, and by using natural dynamic conditions (e.g., holding test insects in outdoor rearing houses or indoor locations receiving natural light), researchers are limited to testing one photoperiod at a time. To simulate a dynamic environment and provide for replicates, we used programmed environmental chambers, which we recognize do not perfectly mimic nature (e.g., light flicker, artificial wavelengths, reduced intensity). We also developed a numerically based system for classifying diapause in tarnished plant bugs and an SAS-based program for identifying and quantifying diapausing and reproductive bugs.

Materials and Methods

Rearing of test bugs. A laboratory colony of tarnished plant bugs was established in February 2002 from insects collected near Starkville (latitude 33.45 N) and Stoneville, Mississippi, at various weed sites, the dominant species being *Erigeron annuus* (L.) or *E. canadensis* L. Artificial diet and methodology developed by Cohen (2000) were used to maintain the colony. To obtain test insects, artificial diet and oviposition gel normally used for colony maintenance were removed, and green bean pods, *Phaseolus vulgaris* L., were inserted into the rearing containers. Green beans are adequate media for allowing tarnished plant bugs to develop the diapause syndrome (Snodgrass 2003). Beans were examined for tarnished plant bug eggs 24 h after presentation and then distributed into 24 Rubbermaid® Servin' Saver (no. 3861; Rubbermaid Home Products Division, Fairlawn, Ohio) containers (1.9 l) with solid plastic tops carved out so that only the top-securing rim remained. The rim was used to secure organdy cloth tops. The containers were loosely filled with shredded paper to reduce opportunities for cannibalism by keeping bugs separated. Green beans were replaced every other day from hatch until dissection.

Environmental cabinets. Two rearing containers were placed in each of 12 environmental cabinets (Model I-36LLX, Percival Scientific, Perry, Iowa) controlled by a Gateway 2000 E-3110 Model Pentium II computer using methodology modified from Wagner & Villavaso (1999a). The computerized setup can be used to mimic dynamic photoperiods and temperatures. For the current study, temperature in all cabinets was fixed at 26.7°C. That temperature is within the 30-year average temperature range of 26.3 to 27.6°C for the months of June, July, and August at Stoneville, Mississippi (Boykin et al. 1995) and within the 25 ± 4°C constant temperature used by Snodgrass (2003) during diapause induction experiments. Constant temperature was used so that the insects would develop diapause morphology at the same rate and could be dissected at the same time. Temperature may play a role in inducing or suppressing diapause in tarnished plant bugs; however, Snodgrass (2003) reared two groups of tarnished plant bugs indoors (2001–2002) under a constant temperature (25 ± 4°C) and natural light and another outdoors under normal winter temperatures and natural light (1999) and noted no difference in percentage of diapause between the two groups. Although not directly comparable because the year differences, the percentage diapause for the three years was reasonably consistent. Similar temperature-based results have been reported for the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) (Wagner & Villavaso 1999b).

The computer dynamically controlled photoperiod. Four 25-W standard incandescent light bulbs (General Electric, Cleveland, Ohio) were turned on and off each day at civil dawn and civil dusk at Starkville, Mississippi. To simulate increasing morning light intensity and decreasing evening light intensity, eight 20-W fluorescent light bulbs (4 Coolwhite and 4 Daylight [General Electric]) were turned on 47 to 71 min after civil dawn and turned off 47 to 71 min before civil dusk, depending on Julian date (JD) (Wagner & Villavaso 1999a).

Experimental procedures. Groups of tarnished plant bugs were assigned to environmental cabinets with dynamic photoperiods for Starkville, Mississippi (latitude 33.45 N) beginning on 4 September, (JD 247, 13.6 h light), 17 September (JD 260, 13.2 h light), and 1 October (JD 274, 12.7 h light). The photoperiods encompassed the dates and day lengths reported by Bariola (1969) and Snodgrass (2003).

Bugs were dissected in phosphate-buffered saline (Wiygul et al. 1982) 12 to 15 days after reaching adulthood. Observations were made under 10-40x magnification on fat body, number of eggs, evidence of mating in females, presence of sperm in the seminal vesicles, and condition of male accessory glands. Lees' (1955) criteria for diapause, used by Bariola (1969) and Snodgrass (2003), are failure of testes or ovaries to enlarge and hypertrophy of the fat body. These are generally accepted criteria for determining adult diapause in most adult insects (Tauber et al. 1986), including the pentatomid *Euschistus consperus* Uhler (Krupke et al. 2001) and the boll weevil (Brazzel & Newsom 1959, Wagner & Villavaso 1999a,b). In adult diapause, females tend not to produce eggs, and in males the accessory glands do not develop because of failure to release necessary hormones (Oberlander 1985). Male tarnished plant bugs with enlarged accessory glands accompanied by white fluid visible in the opaque gland are considered to be reproductive (Snodgrass 2003). Enlarged, chalky-white seminal vesicles indicate that sperm has been produced and tarnished plant bugs with larger seminal vesicles tend to have larger actual numbers of sperm (Villavaso 2004).

Numerical ratings of diapause characteristics were established. Fat body was rated 3 if the abdomen was full of globular whitish fat similar to characterized (Wagner & Villavaso 1999b) and depicted (Spurgeon et al. 2003) in boll weevils, 2 if the globular fat was present in lesser quantities than 3, 1 if a normal complement of fat that did not have the globular appearance was present, and 0 if little or no fat body was apparent.

Seminal vesicles swollen with sperm from the points at which the vasa deferentia entered them to the posterior end were assigned a rating of 3. A rating of 2 was given when the posterior ends and the portions where the vasa deferentia enter the seminal vesicles were distended with sperm, but the midportions were not. A rating of 1 was assigned when sperm was present in the posterior sections of the seminal vesicles, where they run parallel to each other, but not in the horns. Seminal vesicles with no sperm or only a trace detectable in them under the stereoscope at up to 40× were rated 0.

A rating of 3 was assigned to accessory glands that were large and appeared to be fully developed as evidenced by the opaque glands being filled with white fluid. A rating of 2 was assigned to glands larger than 1, but not well developed. Glands rated 2 appeared to be closer in development to those rated 1 than those rated 3, and we considered combining the 1 and 2 classifications into a single classification. A rating of 1 was assigned when the glands had no apparent evidence of development and no manifestation of white fluid in the opaque glands. A rating of 0 was assigned only once when the glands could not be seen.

We had intended to rate testis size, but were unable to establish size categories that could distinguish between diapausing and reproductive males. Instead we determined that a major difference in testes appearance was the presence or absence of a sheath of fatty tissue covering the testes (Snodgrass 2003). This tissue gave the testes a somewhat lighter and more uniform appearance than testes without it, perhaps because it usually obscured the darker testicular lobes that it covered. The fatty tissue tended to be present in males with hypertrophied fat bodies and hypotrophied accessory glands and absent in males with smaller fat bodies and well-developed accessory glands. We rating testes either as having a significant sheath of fatty material or not having it.

Female fat bodies were rated on the same 0 to 3 scale as males. Additionally, we counted the number of mature eggs in the ovaries and examined the seminal depository and genital pouch for evidence of mating, that is, the filling of these organs with white fluid or distension of these tissues (Strong et al. 1970). Females with one or more eggs with a clearly defined operculum were not considered to be in diapause no matter the condition of the fat body.

Statistics. Having established our numerical ratings of diapause characteristics, a simple SAS program (SAS Institute Inc. 1999) was created to classify bugs by the following rules. Males with fat bodies rated ≥2 and accessory glands rated ≤2 and females with fat bodies rated ≥2 and no mature eggs were classified as being in diapause. Status of males with fat bodies and accessory glands rated as 1 and females with fat bodies rated as 1 and no mature eggs was classified as unclear. All other tarnished plant bugs were classified as being reproductive. Least squares means were calculated, and the percentages of tarnished plant bugs classified as being in diapause at each of the three photoperiods were separated by differences of least square means (SAS Institute 1999). Equations and figures were calculated or created using Sigma Plot 2000 for Windows Version 6.0© 1986–2000 SPSS Inc.

Results and Discussion

A total of 1522 tarnished plant bugs were dissected, 772 males and 750 females. We classified 1482 of these insects as either diapausing or reproductive, but we were not sure enough of the status of 40 insects to classify them. Mean percentage diapause for the simulated dynamic photoperiods beginning on hatch dates 4 September, 17 September, and 1 October (JD 247, 260, and 274; day lengths 13.6, 13.2, and 12.7 h) were 15.4%, 57.0%, and 86.0%, respectively (15.4 < 57.0 < 86.0; $P < 0.01$; SAS differences of least squares means). Thus, the incidence of diapause in tarnished plant bugs hatching over a period of 27 days increased almost 6-fold.

Of all insects dissected, 53.4% of males and 52.3% of females were classified as being in diapause ($F = 0.16$; df = 1, 2; $P = 0.6968$). Percentages of males and females in diapause were 17.6 ± 6.1% and 13.3 ± 5.2%; 58.7 ± 4.5% and 55.2 ± 4.5%; and 83.8 ± 4.5% and 88.3 ± 4.55%, for JD 247, 260, and 274, respectively.

The three simulated periods from egg hatch to dissection encompassed JD 247 to 304 and decreasing day lengths of 13.60 to 11.68 h. Curves for JD and day length versus percentage diapause showed large changes in percentage diapause over relatively small changes in date or day length (Fig. 1). Based on Eq. (1), JD of hatch that results in 50% of subsequent adults attaining diapause is 257 (14 September; day length 13.25 h at Starkville, Mississippi). Based on Eq. (2), decreasing day length of hatch that results in 50% of subsequent adults attaining diapause is 13.26 h (closest JD = 257; 14 September), the critical photoperiod. Because the relationship between JD and day length during the test period was strongly linear ($r^2 = 0.999$), similar predictions of critical photoperiod and JD were expected.

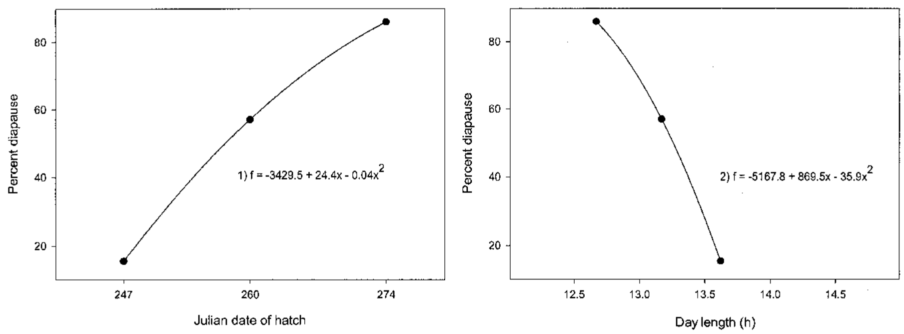

Fig. 1. Julian date and decreasing photoperiod versus percentage diapause in tarnished plant bugs. Day length calculated from civil dawn to civil dusk and simulated to match those occurring at Starkville, Mississippi (latitude 33.45 N), 2002.

$$f = -3429.5 + 24.4x - 0.04x^2 \tag{1}$$

$$f = -5167.8 + 869.5x - 35.9x^2 \tag{2}$$

Bariola (1969) did not report critical photoperiod. The Snodgrass (2003) field study estimated the date for the critical photoperiod to be 12 September, which is very close to our estimate of 14 September, but he assigned the 12.5 h sunrise-to-sunset day length as the critical photoperiod. Using civil dawn to dusk instead of sunrise-to-sunset increased the Snodgrass critical photoperiod to 13.33 h (12 September), which is in close agreement with our estimate of 13.25 h (14 September). Such agreement appears to affirm the validity of our dynamic photoperiod cabinets for simulating field conditions and the use of a civil twilight model rather than the sunrise-to-sunset model. Using Eqs. (1) and (2), the day length for 90% diapause is very near 6 October and a decreasing day length of 12.5 h.

The amount of sperm in the seminal vesicles of males reared in all three photoperiods combined was similar for diapausing and reproductive males. Seventy-one percent (12 of 17) of males with seminal vesicle ratings of 0 were in diapause as were 43% (6 of 14) with ratings of 1; 63% (38 of 60) with ratings of 2; and 56% (383 of 681) with ratings of 3. Eighty-seven percent (383/439) of diapausing males were assigned seminal vesicle ratings of 3, demonstrating that tarnished plant bug males entering diapause produce and accumulate significant amounts of sperm. Both diapausing and reproductive males were caged with females during the predissection period, and many females were inseminated during this period, but we did not determine whether diapausing males were responsible for any of the inseminations. Diapausing male boll weevils can produce significant quantities of sperm and are capable of inseminating females that will, in turn, produce fertile eggs (Villavaso 1981).

The accessory glands of males yielded important information about diapause status (Table 1). A highly significant negative correlation ($P < 0.01$) between fat body and accessory gland ratings was observed (Spearman Correlation coefficient = −0.82; 95% C. I. = −0.79 to −0.85; SAS Institute 1999). Hypotrophied accessory glands (low ratings) generally were associated with hypertrophied fat

Table 1. Relationship between accessory gland size and fat body size in male tarnished plant bugs: numbers of males with associated fat body and accessory gland ratings, Starkville, Mississippi, 2002.

	Accessory gland rating[a,b]				
Fat body rating[a,b]	0	1	2	3	Total
1	1	28	14	270	313
2	0	101	30	17	148
3	0	301	7	3	311
Total	1	430	51	290	772

[a]Accessory glands and fat bodies rated on ordinal scale of 0 to 3, smallest to largest.
[b]Males with fat bodies ≥2 and accessory glands ≥2 were classified as being in diapause.

bodies (high ratings). For example, for males of all three photoperiods combined, 97% (301/311) of accessory gland ratings of 1 were associated with fat body ratings of 3, and 68% (101/148) of accessory gland ratings of 1 were associated with fat body ratings of 2. In contrast, 87% (270/312) of accessory gland ratings of 3 were associated with fat body ratings of 1 (Table 1). We considered the combination of a hypertrophied fat body (2 or 3 rating) and hypotrophied accessory glands (1 or 2 rating) to be simpler and less subjective than the criteria of Lees (1955; hypertrophied fat body and failure of testes to enlarge) to classify diapause in male tarnished plant bugs; therefore, these were the criteria we used. Testes appearance—the presence or absence of sheath of fatty tissue surrounding testes—tended to support the classifications.

No obvious size differences in testis size between diapausing and reproductive males were observed. This observation runs counter to one diapause criterion cited by Lees (1955), which is failure of testes to enlarge. Spermatogenesis had occurred during the predissection period as was evidenced by the large amounts of sperm in the seminal vesicles of most males, whether they were in diapause or not. We do not know if testes would have ceased sperm production and atrophied had we held the males longer than the 2-week predissection period. Nevertheless, the testes of diapausing males could usually be distinguished from those of reproductive males by the presence of a sheath of fatty tissue enclosing them. Testes of 93% of diapausing males were enclosed by the tissue, and 92% of the testes of reproductive males did not have significant amounts of the tissue present.

Of 750 females dissected in the three photoperiodic treatments combined, 440 (58%) harbored no eggs. The percentage of females harboring no eggs at time of dissection increased linearly with increasing fat body size and JD (Fig. 2). At all three JD of hatch, higher fat body ratings were associated with a greater percentage of females with no eggs at time of dissection. When all females were considered, average number of eggs per female was 8.7 ± 0.3 (SE), 3.6 ± 0.3, and 0 for fat body sizes of 1, 2, and 3, respectively (227, 174, and 349 females per fat body size, respectively), demonstrating the negative relationship between fat body size and egg production. Even when considering only those females that produced eggs, eggs per female maintained the negative relationship with fat

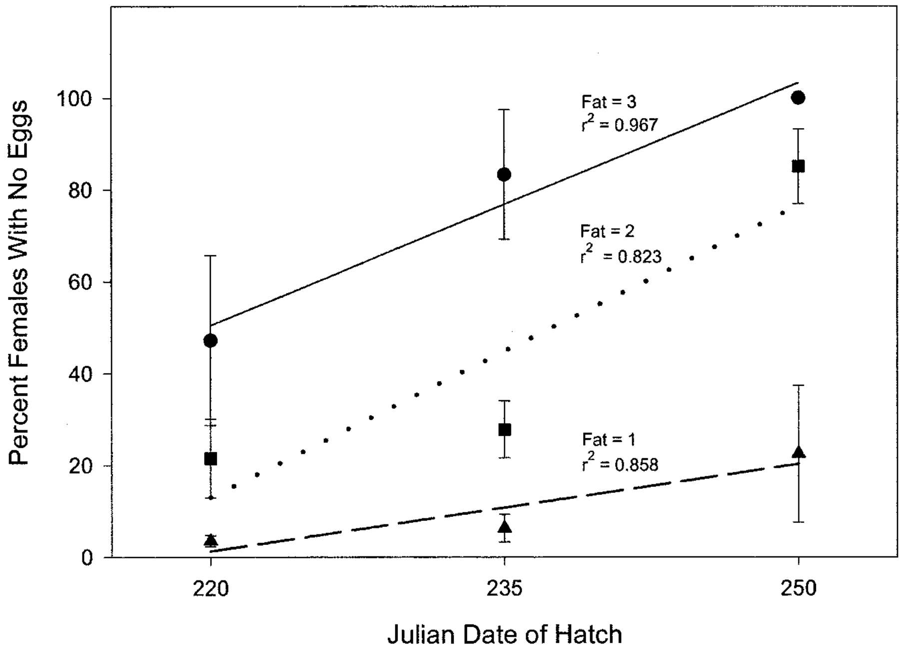

Fig. 2. Percent of tarnished plant bug females with no eggs at time of dissection as related to fat body size (1 to 3, smallest to largest) and photoperiod simulated to match that of Starkville, Mississippi, 2002.

body size seen in Fig. 2. Egg-producing females with fat body ratings of 1, 2, and 3 averaged 9.3 ± 0.3 (SE), 6.8 ± 0.4, and 2.3 ± 0.6 eggs each, respectively (213, 93, and 4 females per fat body size, respectively).

When only egg-harboring females within a fat rating were considered, JD of hatch did not appear to affect number of eggs within females at time of dissection. Within each fat body rating, the average number of eggs within a female remained relatively constant over the three JD of hatch; however, as fat body size increased fewer eggs were present within egg-harboring females (Fig. 3). The very small r^2 values of 0.007 and 0.010 associated with fat body ratings of 1 and 2, respectively, indicate no change in egg numbers within fat ratings over those JD. Though the line (Fig. 3) for fat body rating of 3 had a high r^2 value (0.964), its −0.01 slope was indicative of a very slight decline of 0.3 eggs per female for that rating over the three JD. Thus, though a greater percentage of females harboring no eggs at time of dissection was associated with greater fat body ratings, decreasing photoperiods within those ratings did not appear to have a constraining effect on initial egg production.

Only 4 of 349 females (1.1%) with fat body ratings of 3 harbored one or more eggs, and 10 of 428 females (2.3%) in diapause showed evidence of mating compared with 292 of 310 (94.2%) reproductive females. No unclassified females (0/12) were mated. Whether these insects had been programmed for diapause or reproduction, they did not develop the characteristics we used for classifying either condition. The occurrence of spermatogenesis during diapause develop-

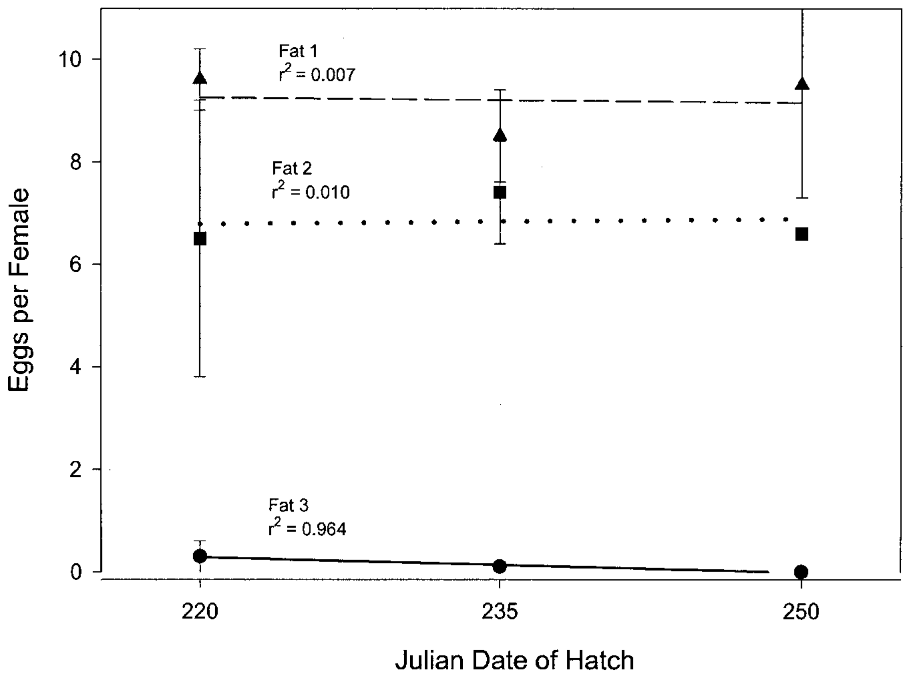

Fig. 3. Average number of eggs at time of dissection (12–15 days after achieving adulthood) within tarnished plant bug females with fat body sizes of 1 to 3 (smallest to largest) and reared at three photoperiods simulated to match those of Starkville, Mississippi, 2002.

ment in tarnished plant bug males and the lack of oogenesis during diapause development in tarnished plant bug females is similar to that observed in the boll weevil (Brazzel & Newsom 1959, Villavaso 1981).

In summary, diapause was induced in tarnished plant bugs held in environmental cabinets with dynamic photoperiods simulating of Starkville, Mississippi beginning 4 September, 17 September, and 1 October (JD 247, 260, and 274, respectively). Diapause morphology was numerically rated, and a SAS program was developed to classify diapausing and reproductive insects based on the ratings. Similar numbers of males and females displayed the diapause syndrome at each photoperiod, and percentage in diapause increased almost 6-fold within the 27-day period separating the earliest and latest hatch dates (15.4%, 57.0%, and 86.0%, respectively). At Starkville, 50% of nymphs eclosing from eggs oviposited about 14 September (13.25 h photoperiod, decreasing) can be expected to enter diapause. A combination of hypertrophied fat body and hypotrophied accessory glands was the criterion we used to classify male diapause. Fat body size was inversely proportional to accessory gland size in both diapausing and reproductive males. Size differences between testes of diapausing and reproductive males were not readily apparent, and almost all males, whether diapausing or reproductive, accumulated significant amounts of sperm in their seminal vesicles. A sheath of fatty tissue covering their testes tended to differentiate diapausing from reproductive males. Hypertrophied fat body and lack of mature eggs were the criteria used for classifying female diapause. The percentage of females harboring no eggs increased, and the number of eggs within females decreased as fat body size increased, but within a fat body rating number of eggs per female was not affected by photoperiod. Almost no diapausing females mated and almost all reproductive females mated.

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